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Polymer ultrathin films with immobilized photosynthetic reaction center proteins

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Abstract

Mixed monolayers of lipids with photosynthetic reaction center proteins (RCs) from *Rb. sphaeroides* and *C. aurantiacus* were studied and the optimum conditions for stable films fabrication were determined. The following synthetic: ACPE, TDA, PDA, DODL and natural lipids: PE, PC were used. The rate of polymerization of the mixed ACPE-RC and TDA-RC monolayers is lower than those for pure lipid-like monomers on the air/water interface but high enough for the fast preparation of the polymer films. The optical and photoelectrical measurements provide evidence for an orientation of RCs (from *Rb. sphaeroides*) on interface. Hydrophilic H-subunit in the mixed ACPE-RCs and DODL-RCs monolayers is preferentially oriented towards water as in the pure RC monolayers. Opposite orientation was found with TDA-RCs and PDA-RCs films. No preferential orientation was found for lipid-RCs (*C. aurantiacus*) monolayers probably because of the low asymmetry of hydrophobic subunits (M and L) of these RCs.

Keywords: Photosynthetic reaction center protein; Lipid-protein monolayer; Absorption spectrum; Optical and photoelectrical property

1. Introduction

The specific interactions between lipids and membrane proteins usually involve a 'boundary lipid' that may be preferentially associated with a given protein. Such a lipid might be expected to act as a specific activator of protein function or to facilitate protein packing in the membrane [1]. RCs are membrane protein-chromophore complexes which provide vectorial photoinduced electron transfer across the cell membrane [2]. The RCs from *Chloroflexus aurantiacus* have two membrane protein subunits (M and L) [3]. The RCs from *Rhodobacter sphaeroides* (wild type) contain three protein subunits (L, M and H). The H subunit is more hydrophilic than L and M subunits and located mainly on the cytoplasmic surface of the membrane-bound LM complex [4]. Langmuir-Blodgett films were obtained from reaction center L-α-dipalmitoylphosphatidylcholine dispersions in [5]. The lipid–protein interactions in phospholipid monolayers containing the antenna protein from *Rb. sphaeroides* were studied using fluorescence spectroscopy and microscopy at the air/water interface [6].

The aim of the present work is the preparation of lipid monolayers with immobilizing photosynthetic RCs (isolated from *C. aurantiacus* and *Rb. sphaeroides*) and their structural-functional characterization using optical and photoelectrical measurements.

2. Materials and methods

2.1. RC preparation

Photosynthetic reaction centers from *Rb. sphaeroides* (wild type) and *C. aurantiacus* were isolated and purified according to the standard procedures [7,8]. The RCs from Rb. sphaeroides were used as a suspension in 10 mM Tris-HCl buffer (1 mM EDTA, 0.1% LDAO, pH 8.0). The RCs from *C. aurantiacus* were suspended in 50 mM Tris-HCl buffer containing 50 mM NaCl, 0.1% LDAO, pH 9.0. The purity of the RC preparations was monitored by determination of the ratio of absorbances at total protein

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band (280 nm) and monomeric bacteriochlorophyll band at 803 nm for *Rb. sphaeroides* RC and at 812 nm for *C. aurantiacus* RC. These ratios were 1.4 for *Rb. sphaeroides* RC and 2.8 for *C. aurantiacus* RC.

2.2. Lipid-protein monolayers

Mixed monolayers were prepared with natural lipids: L-α-phosphatidylethanolamine (PE), L-α-phosphatidylcholine (PC) and specially synthesized lipid-like monomers: *N*-acryloylphosphatidylethanolamine (ACPE), dioctadecyldienoylphosphatidylcholine (DODL), tetracosa-11,13-diinoic acid (TDA), pentacosa-10,12-diinoic acid (PDA). PDA was generously donated by Prof. H. Ringsdorf (University of Mainz, Germany). Lipid-RC monolayers were studied using a commercial film balance (Lauda, Germany). The samples were formed by three methods: (1) the deposition of the preliminary prepared liposome suspension (containing lipids and proteins) on the interface; (2) the deposition of lipid and protein suspensions on air/water interface simultaneously; (3) the deposition of the lipid solution on the interface and following spreading of protein suspension in buffer. Isotherms of surface pressure (π) versus area (A) per molecule in monolayer were recorded under continuous compression of the monolayer. Collapse area (A_c) and the transition area (A_t) per protein molecule were obtained from the surface pressure isotherms at two inflexions of the curves related to the pressure of monolayer collapse (π_c) and the transition between different monolayer states (π_t).

2.3. Monolayer polymerization

Polymerization of monolayers was carried out under UV-illumination (254 nm low pressure mercury lamp, 10 mW/cm^2), in nitrogen under constant pressure and temperature. The rate of polymerization was controlled by decrease of monolayers area and was calculated using equation [9]. A short time of UV-illumination (10–15 min) was not influenced on absorption spectra of RC deposited on solid supports after polymerization.

2.4. Optical and photoelectrical measurements

Multilayer films on quartz and glass covered with SnO_2 were formed by the transfer of lipid-protein monolayers from the air/buffer interface at constant pressure (10–40 mN/m) by the Langmuir-Schaefer technique (horizontal lifting) [10]. Absorption spectra of lipid-RC multilayers were recorded on the spectrophotometer (DU-70, Beckman) in the 200–900 nm range. The technique of photopotential measurement was previously described [11]. Kinetics of the flash-induced $(BChl)_2$ oxidation and reduction were measured at 865 nm. A SnO_2 ultrathin layer deposited on the glass surface served as a negative electrode (grounded to the minus line of the voltage amplifier) and In-Ga paste on top of the RCs film served as a positive electrode.

3. Results and discussion

Mixed monolayers of RCs were prepared using natural lipids: PE, PC, or polymerizable synthetic lipids: ACPE, DODL, TDA, PDA. The isotherms of mixed lipids-RCs and pure RCs monolayers are qualitatively similar and consist of two regions with different linear slopes (Fig. 1) [12]. At low pressures the behavior of these monolayers is defined by detergent molecules lauryldimethylaminoxide (LDAO) which are originally bound to the hydrophobic surface of the protein subunits. This detergent cannot form stable monolayers at the air/buffer interface. A break point is around 32 mN/m, which indicates that at high compression the excess of detergent is removed from the monolayer.

One of the main tasks of this study was the investigation of the influence of lipid/protein ratio on π-A isotherms. Collapse pressure (π_c) for mixed RC-PC and RC-PE monolayers (for both types of RCs) at concentration range 1:26 and 1:100 was more close to corresponding value for pure lipid monolayers. The collapse pressure of mixed monolayers for synthetic lipids (TDA; ACPE) was near to the values for pure protein monolayers.

The area per protein molecule in mixed lipid-RC (*Rb. sphaeroides*) monolayers (except for TDA-RC monolayers) are decreased in comparison with pure protein mono-

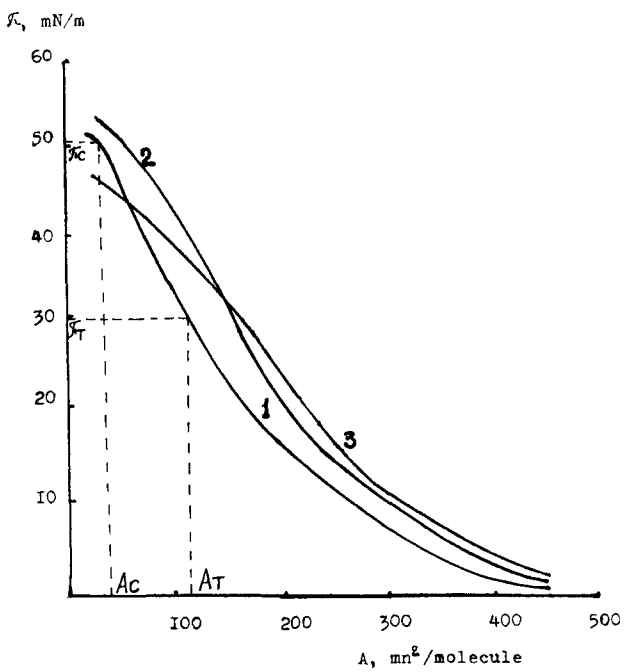

Fig. 1. Surface pressure (π) vs. molecular area (A) isotherms for monolayers of RCs (wild type) (1), ACPE-*Rb. sphaeroides* RCs (10:1) (M/M) (2), PC-*Rb. sphaeroides* RCs (100:1) (M/M) (3) at the interface of 10 mM phosphate buffer, pH 7.0, 20°C.

layers. The area per protein molecule in mixed lipid-RC (*C. aurantiacus*) monolayers was constant. This difference in area per protein molecule is related to the different protein structure orientation on interface. The monolayers characteristics of mixed lipid-Rb. sphaeroides RC monolayers are dependent on the structure of lipids (Table 1). The pronounced increase of the area per RCs was observed in the mixed monolayers of TDA-RCs (*Rb. sphaeroides*) that can be explained by very strong interaction between RCs and TDA. Thus, TDA stays with RC up to the monolayer collapse. The high decrease in the area per RCs in the mixed monolayers of RCs (*Rb. sphaeroides*) with PC, PE and ACPE can be explained by weak lipid–protein interactions. Probably some protein squeezes to buffer subphase, but lipid stays in monolayers. There absolutely no changes in the area per protein in the mixed monolayers of RCs from *C. aurantiacus* and various lipids. Probably lipid leaves the monolayer, but RC stays, because of the weak lipid–protein interactions.

The best deposition of mixed monolayers for the following optical and photoelectrical measurements should be done under optimal conditions of the aqueous subphase. For this purpose the mixed standard lipid-RC (26:1) (M/M) monolayer characteristics were analyzed under different conditions of the aqueous subphase. The optimum conditions for lipid (PC, PE):RC (*Rb. sphaeroides, C. aurantiacus*) (26:1) (M/M) monolayer preparations appear to be 1–10 mM phosphate buffer as the aqueous subphase, pH 7.0 and temperature of less than 20°C. The deposition experiments were carried out under the above-mentioned conditions.

Lipid-like monomers in the mixed lipid-protein monolayers were polymerized by UV-irradiation for the stabilization of proteins in lipid matrix. ACPE has polymerizable groups in the hydrophilic parts of molecules, whereas DODL, TDA and PDA have polymerizable groups in the hydrophobic parts. Polymerization of diinoic acid was controlled by absorption spectroscopy. Reaction of polymerization is characterized by formation of the intermediate which has an absorption band at 630 nm (so called 'blue' polymer). The final product has two peaks of absorption 530 nm and 540 nm (so-called 'red' polymer) (Fig. 2), that is in a good agreement with the work [13]. Note that RCs absorption is too weak to show up in a spectrum before polymerization. The rates of polymerization of lipid-like monomers in mixed monolayers were lower as compared with corresponding values for pure lipid-like monomers. The rate of polymerization was $1.5 \cdot 10^{-3}$ s^{-1} for TDA-RC (*Rb. sphaeroides*) (100:1) (M/M) at constant pressure 20 mN/m, whereas the corresponding value for pure lipid-like monomer was $16 \cdot 10^{-3}$ s^{-1}. There were almost no changes in area for mixed ACPE-RC (*Rb. sphaeroides*) (10:1) (M/M) monolayer at 20 mN/m. The rate of polymerization of ACPE in mixed ACPE-RC (*C. aurantiacus*) monolayer (10:1) (M/M) at constant pressure 30 mN/m was 10^{-4} s^{-1}, the rate for pure ACPE was $2 \cdot 10^{-4}$ s^{-1}. The decrease in the rate of polymerization was connected with protein incorporations into the lipid matrix, i.e. the polymerizable lipid-like monomers were diluted by unpolymerizable proteins.

To evaluate structural and functional integrity of lipid-RC monolayers, the spectral and photoelectrical properties of their multilayer films were investigated. It was shown that the lowest surface pressure for monolayer transfer from the buffer subphase onto a solid support was 10 mN/m. The transfer coefficient was about 70 ± 5% at this pressure. The optimum pressure for lipid-RC monolayer transfer was 30 mN/m by the Langmuir-Schaefer method. In this case the transfer coefficient was approximately 95 ± 5%.

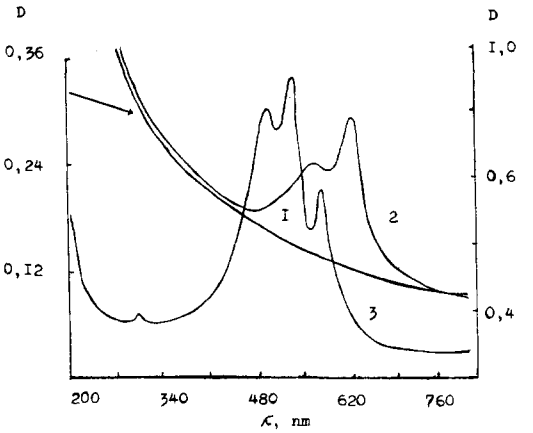

Fig. 2. Absorption (D) vs. wavelength (λ) for 15 monolayers TDA-*Rb. sphaeroides* RCs (100:1) (M/M) deposited onto transparent support before TDA polymerization (I), and after polymerization with formation of 'blue' polymer (2) and 'red' polymer (3). Subphase was 10 mM phosphate buffer, pH 7.0, 20°C. The transfer pressure is 30 mN/m.

Table 1
Monolayer characteristics of mixed monolayers

Content (M/M)	A_c (nm^2/mol)	π_c (mN/m)
Rb. sphaeroides RC	63	50
PC	0.56	46
RC/PC 1:10	27	44
RC/PC 1:26	25	45
RC/PC 1:100	22	44
RC/PC 1:200	26	44
PE	0.56	45
RC/PE 1:26	25	47
ACPE	0.80	50
RC/ACPE 1:10	32	52
TDA	0.35	39
RC/TDA 1:100	94	52
C. aurantiacus RC	20	52
RC/PC 1:200	20	44
RC/PE 1:200	20	50
RC/ACPE 1:10	20	52

Ac — area per molecule of lipid (for pure lipid monolayers) or of protein (for mixed lipid-protein monolayers) at the collapse pressure (π_c) of lipid-RC films at 10 mM phosphate buffer, pH 7.0, 20°C.

Spectra of lipid-RC films are similar to the native spectra of RC, there is a small blue shift of the band of dimeric bacteriochlorophyll typical for the dry thin films of the pure RCs. The presence of lipids in the monolayers had not altered the optical properties of the RC in the range 600–900 nm (Fig. 3). The lipid-RC films were submerged into an aqueous solution of sodium ascorbate (1 mM) and dichlorophenylindophenol (0.1 mM). Under these conditions, the reduction of dimeric bacteriochlorophyll was observed. The optical absorption of monomeric bacteriochlorophyll versus the number of lipid-RC monolayers (transferred onto transparent supports) is linear, indicating that monolayers were transferred quantitatively. Monomeric bacteriochlorophyll in the lipid-RC film was found to retain 60% absorbance after 5 days storage [12]. Probably, the several monolayers on top of the film undergo some destruction by oxygen and these monolayers further served as protection for the underlying monolayers.

A lipid-RC film with 10 monolayers of stearic acid deposited on top and bottom of the film has almost the same optical properties after 14 days storage (Fig. 4). The bacteriochlorophyll dimers in this film were found to be slightly oxidized. Monolayers of stearic acid protected protein molecules from damage. The time of dark reduction of $(BChl)^{+2}$ was 60.5 ± 3 ms. This value was found to be nearly the same for the charge recombination of $(BChl)^{+2}$ with the primary quinone acceptor Q-A in solution of RC from *Rb. sphaeroides* (approx. 90 ms). The photopotential measured for the multilayer films of mixtures of RCs with ACPE, TDA and PDA was about 1–2 mV per single monolayer that corresponded to those for the pure RC films [14]. DODL-RC films had about 2.5–3 mV per single monolayer. It can be explained as the higher degree of protein orientation in DODL-RC monolayers in comparison with other mixed monolayers.

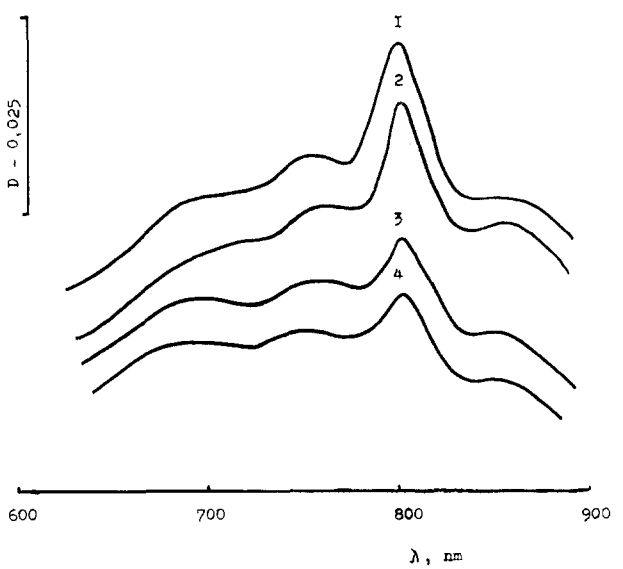

Fig. 3. Absorption spectra of 15 monolayers ACPE-*Rb. sphaeroides* RCs (10:1) (M/M) deposited onto transparent support (I), after submergence into an aqueous solution of sodium ascorbate (1 mM) and dichlorophenylindophenol (0.1 mM) (2), the same film after 5 days (3) and 20 days (4) storage. Subphase 10 mM phosphate buffer, pH 7.0, 20°C. The transfer pressure is 30 mN/m.

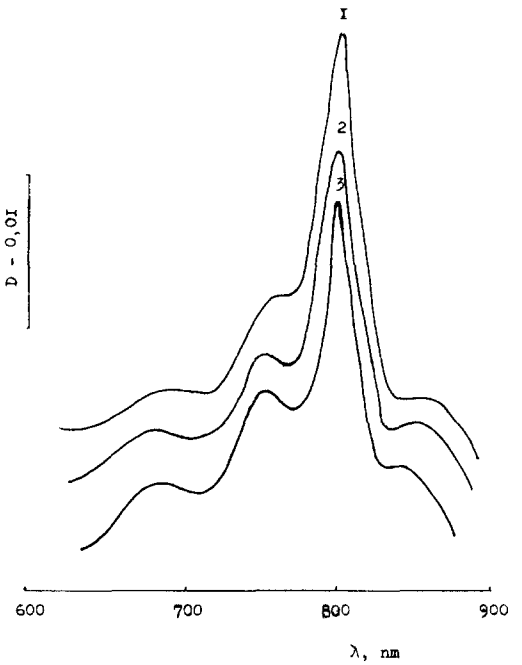

Fig. 4. Absorption spectra for 15 monolayers ACPE-*Rb. sphaeroides* RCs (10:1) (M/M) deposited onto transparent support with 10 monolayers of stearic acid on top and bottom of the film (I), after submergence into an aqueous solution of sodium ascorbate (1 mM) and dichlorophenylindophenol (0.1 mM) (2), after 14 days storage (3). Subphase was 10 mM phosphate buffer, pH 7.0, 20°C. The transfer pressure was 30 mN/m.

The sign of the photopotential (plus on the SnO_2 support for monomeric and polymeric films of ACPE-RC and DODL-RC from *Rb. sphaeroides* provides evidence that the H-subunit in such monolayers faces preferentially the air/buffer interface so as to interact with water (Fig. 5a,b). The same situation was observed in pure RC films [14]. A different situation was observed with TDA-RC films and PDA-RC films, having the minus sign on the SnO_2 electrodes, indicating that the H-subunit is preferentially oriented towards air (Fig. 5c).

We could not observe a photopotential of RC (*C. aurantiacus*) multilayers, although the absorption spectra of films had the same bands for RC from *C. aurantiacus* as in the buffer suspension. Consequently, RC from *C. aurantiacus* had not preferential orientation on air/buffer interface and hence on solid supports.

The results show that transferred multilayer films retain RC activity (in the case of *Rb. sphaeroides*) with the RCs having net orientations differing by 180° depending on the nature of the lipid.

Summarizing, monolayers composed of lipids and RCs of the photosynthetic bacteria *Rb. sphaeroides* (wild type) and *C. aurantiacus* were characterized using surface pressure-molecular area isotherms and variable concentrations

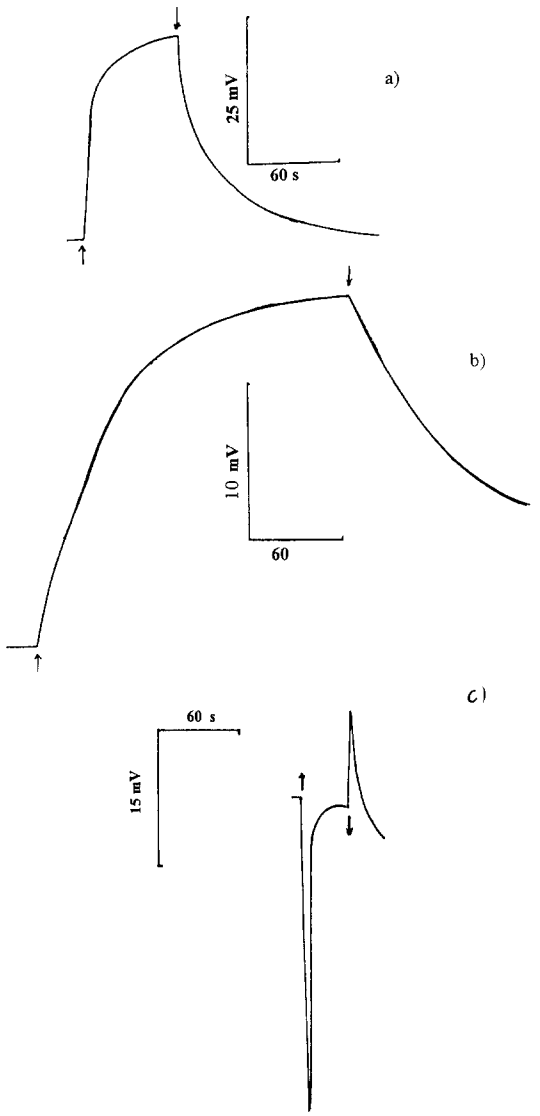

Fig. 5. Photoinduced electrical response of a multilayer film composed of 15 monolayers of ACPE-*Rb. sphaeroides* RCs (10:1) (M/M) with 10 monolayers of stearic acid on top and bottom of the film (a), of 10 monolayers of the ACPE-RC *Rb. sphaeroides* (10:1) (M/M) after polymerization (b) and of 25 monolayers of the TDA-RC *Rb. sphaeroides* (100:1) (M/M) with 10 monolayers of stearic acid on top and bottom of the film (c) under continuous excitation (light on ↑, light off ↓).

of subphase buffer, pH and temperature of the aqueous subphase. The monolayer characteristics were depended on charges of the lipids in the monolayers. The rates of polymerization of lipid-like monomers in mixed monolayers were lower as compared with the corresponding values for pure lipid-like monomers. Monolayers of stearic acid deposited on top and bottom of the film protected protein molecules from damage in the air. The H-subunit in ACPE-*Rb. sphaeroides* RC films and DODL-RC films was oriented on the air/buffer interface so as to interact with water. The same situation was observed in pure RC films. A different situation was seen with diinoic acid-*Rb. sphaeroides* RC films. Here, the H-subunit of RC *Rb. sphaeroides* had net orientations different by 180° depending on lipid structures.

Acknowledgements

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